Progression and regression of myointimal hyperplasia in experimental vein grafts depends on platelet-derived growth factor and basic fibroblastic growth factor production

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Purpose: **The factors that lead to myointimal hyperplasia (MH) in arterial vein grafts (AVGs) are unknown. Platelet-derived growth factor (PDGF) and basic fibroblastic growth factor (bFGF) are two powerful mitogens for smooth muscle cells that have been implicated in the genesis of MH. The aim of this study was to analyze the correlation between progression and regression of MH and production of PDGF and bFGF in experimental vein grafts.**

Materials: **In 64 inbred Lewis rats, a 1-cm segment of inferior vena cava was inserted at the level of the abdominal aorta. The segments of inferior vena cava were obtained from syngenic rats. In 48 rats, the AVG was explanted 3 days (n = 8), 7 days (n = 8), 4 weeks (n = 24), and 12 weeks (n = 8) after surgery. In 16 rats the vein graft was explanted after being in the arterial system for 4 weeks and was reimplanted as a venous-venous bypass in syngenic Lewis rats. Reimplanted vein grafts (RVGs) were explanted 2 weeks (n = 8) and 8 weeks (n = 8) later. Grafts were analyzed by light and electron microscopy, morphometry, and histochemistry, and were put in organ culture to assess PDGF and bFGF production and mitogenic activity.**

Results: **We observed MH formation in AVGs and MH regression in RVGs ($p < 0.001$). PDGF and bFGF production correlated with the degree of MH ($p < 0.01$). Histochemistry showed PDGF and bFGF in the area of MH in AVG, which disappeared in RVG. Conditioned media from AVG had greater mitogenic activity than RVG or control veins.**

Conclusions: **MH formation and regression in experimental vein grafts correlate with PDGF and bFGF production. (J VASC SURG 1996;23:568-75.)**

Despite the durability of the autogenous saphenous vein graft, the incidence of thrombosis at the end of the first year in the coronary and lower-extremity circulation ranges from 20% to 50%.[1-3] Many of these failures result from the development of vein graft stenosis, which is often caused by myointimal hyperplasia (MH).[4,5]

In 1906, Carrel and Guthrie[6] observed that vein grafts, once implanted in the arterial circulation, adapt by undergoing wall thickening. The formation of intimal thickening depends on smooth muscle cell (SMC) proliferation and migration and on matrix synthesis.[7] The factors that lead to MH in arterial vein grafts (AVGs) are not well-defined. Several hemodynamic factors that could explain vein-graft MH have been identified: reduced flow velocity and shear stress,[8] tangential stress,[9] and increased deformation of the vein wall in the circumferential direction.[10-12] Preexisting vein disease could be responsible for MH in some cases.[13] The biologic mechanisms leading to MH in vein grafts implanted in the arterial circulation, however, are not known.

Two of the best-analyzed mitogens for SMCs are platelet-derived growth factor (PDGF)[14] and basic fibroblastic growth factor (bFGF).[15] PDGF was origi-

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nally identified from human platelets, but other types of cells were found to be able to release PDGF-like molecules, including endothelial cells (ECs) and SMCs. Pure PDGF was described as a 27- to 31-KD glycoprotein that was thought to exist as a dimer of two distinct but highly similar polypeptides, which were termed A chain and B chain.[16,17] PDGF is a major mitogen for connective tissue cells.[18,19] bFGF is a member of the heparin-binding mitogens and is ubiquitously distributed.[15]

The principal animal model that has been used to study MH is balloon angioplasty of the normal rat artery. Ferns et al.[20] demonstrated that the development of an intimal lesion induced by intraarterial balloon catheter was reduced 40.9% by administration of the antibody to PDGF. Analysis of segments of primary and restenotic human lesions obtained with in-situ hybridization after atherectomy has demonstrated increased expression of PDGF.[21] Lindner and Reidy[22] showed that bFGF is a potent pharmacologic stimulant of SMC growth in injured arteries. They demonstrated that the administration of a blocking antibody to bFGF inhibits the proliferation of SMCs in balloon-injured arteries by 80%. The results of their study suggest that endogenous bFGF is a major mitogen that controls the growth of vascular SMCs after injury.

Theoretically, PDGF and bFGF could be responsible for MH formation in AVGs. The purpose of this study was to determine the production of PDGF and bFGF by vein grafts when inserted into the arterial circulation and by AVGs when reimplanted in the venous system.

MATERIAL AND METHODS

Experimental design and operative procedures. Male inbred Lewis rats with an average weight of 250 gm were used in the study. In 64 rats, 1-cm segments of inferior vena cava that had been obtained from syngenic rats were implanted at the level of the abdominal aorta. In 48 rats, the AVG was explanted 3 days (n = 8), 7 days (n = 8), 4 weeks (n = 24), and 12 weeks (n = 8) after surgery. In 16 rats the vein graft was explanted after 4 weeks in the arterial system and was reimplanted as a venous-venous bypass in syngenic Lewis rats. Reimplanted vein grafts (RVG) were explanted 2 weeks (n = 8) and 8 weeks (n = 8) later. At the time of harvest, segments of similar dimensions of endogenous inferior vena cava aorta above and below the graft were removed.

The animals were anesthetized with intramuscular xylazine (3 mg/kg) and intramuscular ketamine (50 mg/kg) supplemented by intraperitoneal ketamine for maintenance. Surgery was performed with an operating microscope (Zeiss OPMI 7-D; Carl Zeiss, Inc.; Thornwood, N.Y.). A 1-cm segment of supradiaphragmatic inferior vena cava was obtained from donor rats. The chest was opened, and the supradiaphragmatic portion of the inferior vena cava was identified and excised. The rat was killed with an overdose of anesthetic. The vein was kept in Dulbecco's modified Eagle's medium (DMEM) for approximately 20 minutes while the receiving rat was anesthetized, the abdominal aorta identified, and both proximal and distal control specimens obtained. The vein grafts were inserted with end-to-end anastomoses using 10-0 nylon sutures (Ethicon Inc.; Somerville, N.J.) in a continuous fashion.

In the RVG group, the AVG was harvested after being in the arterial system for 4 weeks and was reimplanted with the same microsurgical procedures and methods at the level of the left iliac vein with end-to-end anastomoses. The patency of the grafts was evaluated at the time of surgery and was evaluated thereafter by weekly palpation of the femoral pulse and inspection for leg swelling.

Animal care complied with the "Principles of Laboratory Animal Care" as formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 86-23, revised 1985).

At the time of harvest, the abdomen and chest were opened with the animal under deep anesthesia. The aorta or the left iliac vein above and below the graft, the vein graft, and a segment of the same dimensions of the supradiaphragmatic inferior vena cava were exposed and excised. The rat then was killed with an overdose of anesthetic. Three grafts from each group were divided in segments and processed for light and electron microscopy and histochemistry. Five grafts for each group were studied for PDGF and bFGF.

Histology. Segments of grafts were fixed in formaldehyde. Following standard procedure, the specimens were stained with hematoxylin and eosin. Intimal and medial areas were calculated by videomorphometry (Quantimet 500) at different levels. For scanning electron microscopy, specimens were fixed in 2.5% glutaraldehyde made up in 0.1 mol/L cacodylate buffer (pH, 7.2), rinsed several times, and left for 1 hour in the same buffer. Next, the specimens were fixed in osmium 1% for 1 hour, dehydrated in ascending concentrations of ethanol (15% to 100%), critical point–dried in CO_2, mounted in specimen stubs, and sputter coated with gold-palladium according to standard technique. All specimens

Table I. Intima-media area ($\mu m^2 \times 10^5$)

Control vein	1.9 ± 0.8
AVG	
3 days	6.4 ± 2.4
7 days	13.1 ± 4.1
4 weeks	16.8 ± 4.4
12 weeks	17.5 ± 3.6
RVG	
2 weeks	15.0 ± 6.0
8 weeks	5.9 ± 3.3

were examined in a scanning electron microscope (Hitachi S 570; Tokyo) at the accelerating voltage of 15 kV.

Histochemistry. Tissues were prepared for immunohistochemical analysis of bFGF, PDGF, and α-actin with frozen section techniques to guarantee accessibility to intracellular antigens as previously described.[23] Briefly, individual sections were made within the midportion of the vein graft and control vein with a 1720 Digital MGW Lauda cryostat (Leitz; Wetzlar, Germany) and placed on gelatin-coated glass slides. Sections were fixed in cold methanol, rehydrated, absorbed with normal mouse serum to block nonspecific binding of reagents, and treated with 4% H_2O_2 to eliminate endogenous peroxidase. Sections were covered with primary mouse monoclonal antibodies anti-bFGF and anti–α-actin (Sigma Chemical Co; St. Louis) and rabbit polyclonal antibodies anti-PDGF, followed by goat anti-mouse immunoglobulin G and goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Sigma) for amplification. Peroxidase localization was visualized with diaminobenzidine.[23] Control sections were treated in an identical manner except that nonspecific immunoglobulin G antibodies were used.

Organ culture. The arterial and vein grafts and the aortic segment above and below the graft (five grafts from each group) were opened longitudinally and rinsed thoroughly for 10 minutes with DMEM supplemented with antibiotics (gentamicin 200 μg/ml, streptomycin 100 μg/ml, penicillin 100 IU/ml). The specimens were placed in 48-well Costar (Costar Corp.; Cambridge, Mass.) tissue culture plates for organ culture. The tissue was incubated for 5 days at 37° C in a 5% CO_2 atmosphere. Aliquots of conditioned media were collected at 72 hours and centrifuged for 5 minutes at 15,000 rpm, and the supernatant was stored at −80° C for assay of mitogenic activity and assay for PDGF and bFGF release.

Assay for mitogenic activity. Swiss 3T3 cells were plated in 96-well plates (Falcon Plastic, Oxnard, Calif.) at a density of 4×10^4 cells/ml in 200 μl DMEM supplemented with 0.1% fetal calf serum. Seventy-two hours later conditioned serum free media from AVG and RVG were added to Swiss 3T3 cells (20 μl). Positive control specimens received an equivalent volume of DMEM plus human recombinant bFGF (20 ng/ml) or human recombinant PDGF (50 ng/ml; Boehringer Mannheim; Mannheim, Germany); negative control specimens received only DMEM. After 2 days, tritiated thymidine (0.5 μCi per well plate) was added, the cultures were incubated for 18 hours and collected on Skatron filters (Skatron Instruments; Sterling, Va.) for radioactivity determination in an LKB (LKB Instruments, Inc.; Gaithersburg, Md.) scintillation counter.

Reduction of mitogenic activity. Measurement of Swiss 3T3 cells' DNA synthesis-stimulating activity of the conditioned media from AVG and RVG was repeated in the presence of an excess of antibodies to PDGF AA and PDGF BB (50 μg/ml; Genzyme Co; Boston, Mass.) and in the presence of monoclonal antibody to bFGF (produced in our laboratory). Tritiated thymidine was again added and cultures were incubated for 18 hours. After further processing, the radioactivity was measured.

Assay of PDGF and bFGF in conditioned media. The presence of bFGF and PDGF molecules in serum-free conditioned media from AVG, RVG, and aortic segments was determined by inhibition antibody-binding assay. A dilution of anti-bFGF mouse monoclonal antibody that showed 50% maximal reactivity against bFGF was incubated with various dilutions of conditioned media in 400 μl tubes precoated with phosphate-buffered saline (PBS) gelatin 2%. After 20 hours of incubation at 4° C, *Staphylococcus aureus*–protein A was added, and the immunoaggregates were removed by centrifugation. The residual antibody-binding activity in the supernatant was measured by enzyme-linked immunosorbent assay. Plastic wells (96 wells, Falcon Plastic) were coated with bFGF (10 ng per well) for 8 hours at 4° C. Plates then were washed twice with PBS solution and saturated with PBS gelatin 1% for 2 hours at 37° C. Washed wells were then filled with 50 μl per well of supernatant obtained after immunoprecipitation. After 2 hours of incubation at 37° C, the wells were washed with PBS gelatin 0.1%. Peroxidase-labeled goat anti-mouse immunoglobulin antibody was added. After 60 minutes of incubation at 37° C, the plate was washed three times in PBS gelatin 0.1% and once in distilled water. Finally o-phenylenediamine dihydrochloride 0.4 mg/ml (Sigma Chemical Co.; St. Louis) was added as substrate for the enzyme. Bound specific antibody was quantitatively measured

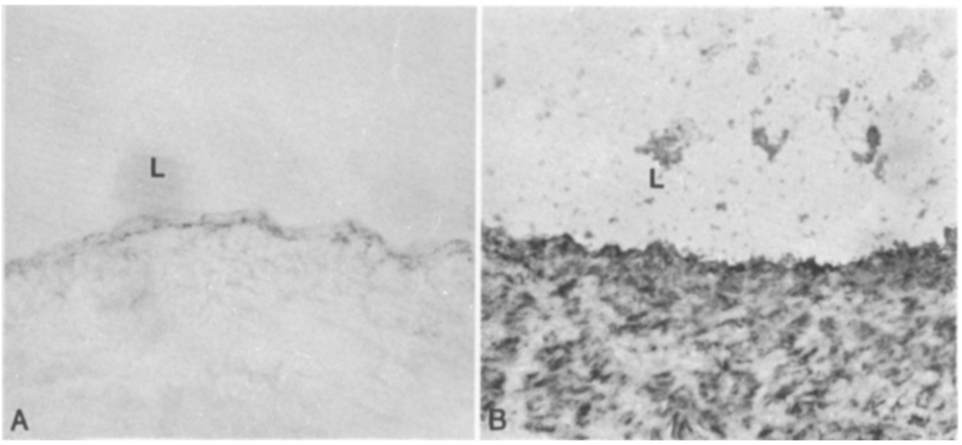

Fig. 1. Transverse section of control vein **(A)** and of AVG **(B)** 4 weeks after surgery (original magnification, ×400). Muscle-specific α-actin demonstrated that SMCs formed the area of MH of AVG. *L*, lumen.

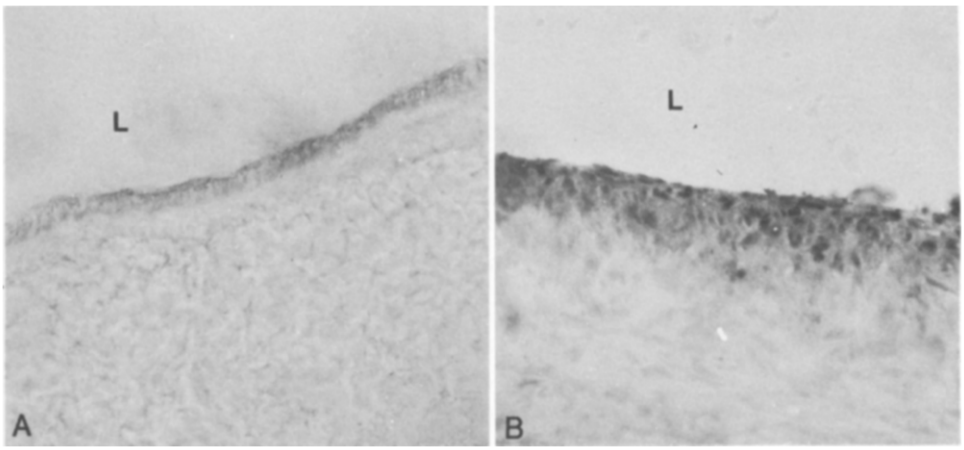

Fig. 2. Localization of bFGF in control ungrafted vein **(A)** and in AVG **(B)** 4 weeks after surgery (transverse section; original magnification, ×400). **A** shows no localization detectable for bFGF in control ungrafted veins. **B** shows localization of bFGF in SMCs of AVG. *L*, lumen.

by optical density reading at 492 nm with a spectrophotometer (Beckman Instruments, Inc.; Palo Alto, Calif.). The amount of bFGF in the conditioned media was determined with a reference curve obtained with known quantities of human recombinant bFGF. We used mouse anti-bFGF monoclonal antibody as the positive control and antibody without specificity as the negative control. Similar experiments were used to determine the presence and amount of PDGF in the conditioned media.

Statistical analysis. We used the χ^2 test, analysis of variance, and Student's t test where appropriate. Data were expressed as mean ± SD. Differences were considered significant at the 5% critical level.

RESULTS

Structural changes. At harvest AVGs were firm and rigid when compared with control veins. Microscopically, intact ECs were present on the surface of AVG and RVG and in the control vein. Scattered platelets and leukocytes adhered to ECs. MH was present in all arterialized vein grafts. Large amounts of connective tissue and several layers of SMCs were also present. In the RVGs, connective tissue was preponderant over SMCs. Control veins consisted of a single layer of ECs and a few layers of SMCs.

Table I shows the total area of intima and media (μm^2) of AVGs, RVGs, and control veins. In AVGs,

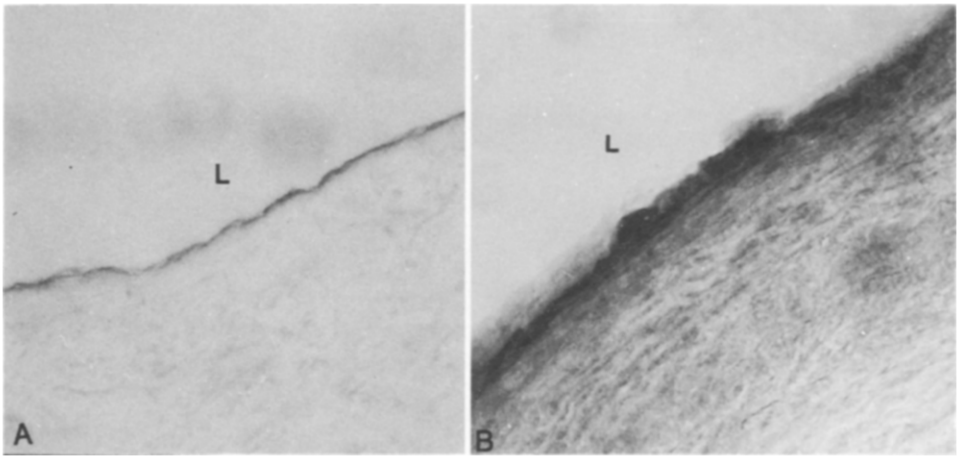

Fig. 3. Localization of PDGF in control ungrafted vein **(A)** and in AVG **(B)** 4 weeks after surgery (transverse section; original magnification, ×400). Localization of PDGF in SMCs of AVG is evident. *L*, lumen.

intima-media area increased significantly with time. Two weeks after reimplantation, RVGs showed a decrease in the total area of intima and media when compared with the AVGs; the difference became significant after 8 weeks ($p < 0.05$).

Histochemistry. Histochemistry showed that the area of MH in AVG was composed of SMCs that were positive for α-actin (Fig. 1). PDGF and bFGF, which were absent in both the control veins and RVGs, were present in the area of MH in AVGs (Fig. 2 and Fig. 3).

Mitogenic activity of conditioned media. The conditioned media from AVG had more mitogenic activity than did the conditioned media from RVG or control vein ($p < 0.001$). This increased production of mitogens could stimulate the growth of SMCs and lead to MH.

Reduction of mitogenic activity by anti-bFGF anti-PDGF antibodies suggests that the mitogenic activity is caused by that particular growth factor. Addition of a mixture of anti-PDGF AA and anti-PDGF BB antibodies to the medium of 3T3 cell cultures exposed to conditioned medium from AVG decreased the uptake of tritiated thymidine by an average of 25% ($p < 0.001$; Fig 4). Addition of an excess of anti-bFGF antibody to the medium of 3T3 cell cultures exposed to conditioned medium from AVG decreased the uptake of tritiated thymidine by an average of 55% ($p < 0.001$; Fig 5).

PDGF and bFGF assay by enzyme-linked immunosorbent assay. Table II shows the production of PDGF and bFGF by AVG, RVG, and control vein. AVG produced a higher quantity of growth factors ($p < 0.01$).

DISCUSSION

Once inserted in the arterial circulation, vein grafts show diffuse MH and wall thickening. In the rabbit experimental model, MH did not develop in vein segments implanted in the venous circulation, which suggests that the arterial environment and not surgical manipulation is required for the formation of this lesion.[24]

Davies et al.[24] performed common carotid vein bypass in rabbits. MH developed in all grafts and regressed significantly after the vein grafts were reimplanted in the venous circulation. In a similar study, Fann et al.[25] showed that MH in vein grafts regresses significantly when a graft implanted in the arterial circulation for 12 weeks is reimplanted in the venous system and harvested after an additional 12 weeks. Our study revealed similar findings. How does arterial flow influence the formation of MH? Berguer et al.[10] showed that in AVGs, MH is more evident in areas of low flow velocity. Similar results were found by Dobrin et al.,[11] who found that MH in AVGs was associated with low flow velocity and a high degree of circumferential deformation. Morinaga et al.[26] found an increased degree of MH in AVGs implanted under low flow conditions that regressed once the grafts were reimplanted in a system with normal parameters of flow. Experiments with prosthetic arterial grafts[27,28] showed a reverse relationship between high flow and high shear stress and the degree of MH.

We found that vein grafts, once inserted in the arterial circulation, develop MH and produce a large quantity of growth factors, including PDGF and

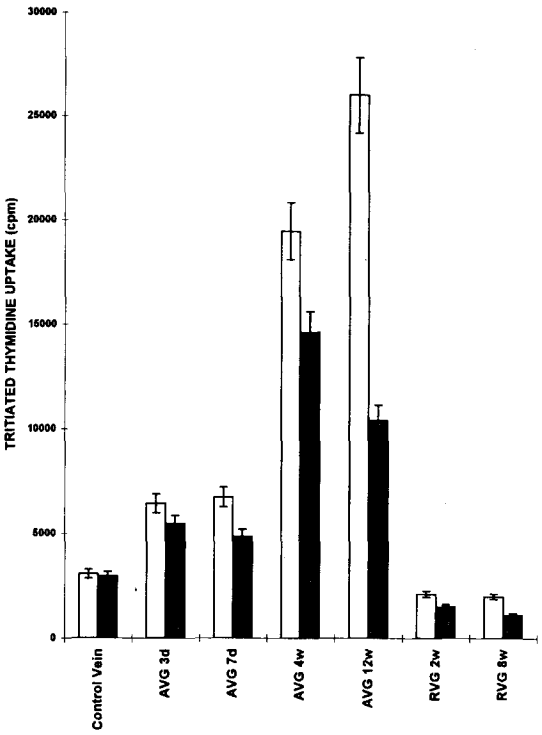

Fig. 4. Mitogenic activity expressed for 1 cm^2 of graft surface *(empty columns)* and its reduction by anti-PDGF antibody *(full columns)*.

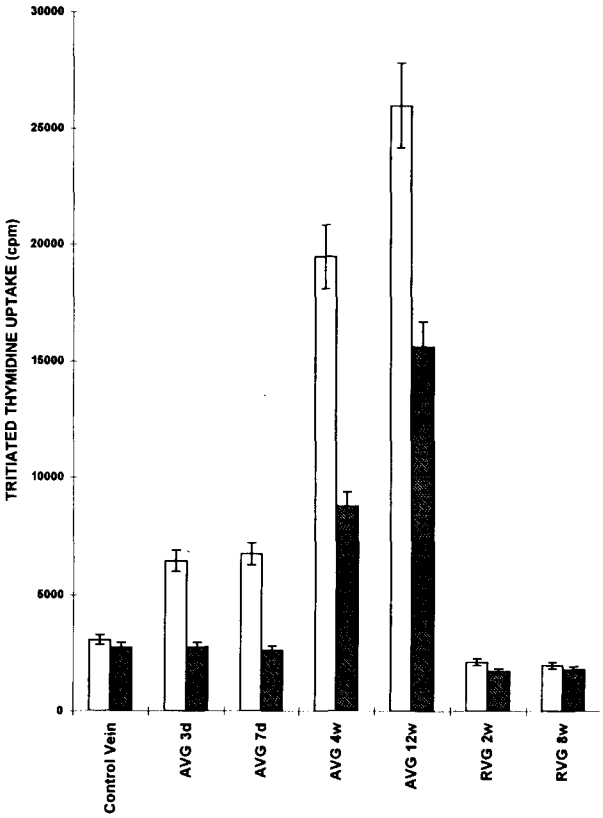

Fig. 5. Mitogenic activity expressed for 1 cm^2 of graft surface *(empty columns)* and its reduction by anti-bFGF antibody *(full columns)*.

bFGF. Hypothetically, vein grafts develop MH to adapt to the new hemodynamic system. Increasing shear stress has been shown to promote the release of PDGF and bFGF by ECs and SMCs. Hsieh et al.[29] showed that shear stress increases PDGF mRNA levels in ECs. Maiek et al.[30] demonstrated that laminar shear stress induced a significant increase of PDGF B and bFGF mRNA levels in ECs. They found that expression of each peptide growth factor gene is differentially regulated by fluid shear stress. Resnick et al.[31] found that shear stress determines expression of PDGF B in ECs by inducing transcription factors that interact with a common promoter element. The signal promoting that increases production of PDGF is ill-defined. Shear stress induction of PDGF and bFGF expression may depend on signal transduction mechanisms involving protein kinase C activation. Previous work has shown that shear stress stimulates phosphoinositide turnover in ECs, producing the second messengers inositol triphosphate and diacylglycerol, which stimulate protein kinase C activity.[32]

These growth factors may be responsible for MH through a paracrine/autocrine mechanism. Although macrophages and leukocytes may produce PDGF and bFGF, neither of these cell types appeared to account for the differences observed in PDGF and bFGF release between vein grafts and control veins; ECs and SMCs probably are the source of these growth factors. It is evident that ECs and SMCs can release growth factors and sustain their own growth.

Table II. Growth factor production (ng/cm^2/72 hr)

	PDGF	*bFGF*
Control vein	11 ± 3	55 ± 7
AVG		
3 days	33 ± 5	128 ± 9
7 days	45 ± 7	153 ± 11
4 weeks	69 ± 8	295 ± 20
12 weeks	65 ± 9	340 ± 18
RVG		
2 weeks	39 ± 9	86 ± 10
8 weeks	13 ± 3	92 ± 12

PDGF and bFGF most likely represent a central factor in the development of MH in AVGs. Experimental studies support this hypothesis. Several re-

ports have demonstrated increased expression of PDGF in association with naturally occurring atherosclerosis, experimentally induced MH, and MH that was associated with failure of vascular grafts. PDGF BB, when infused into rats subjected to balloon carotid injury, produced a two- to threefold increase in medial SMC proliferation. More importantly, PDGF BB greatly increased (20-fold) the intimal thickening and migration of SMCs from the media to the intima during the first 7 days after injury.[33] Administration of polyclonal antibodies to PDGF resulted in a 40.9% reduction in the area of MH induced by intraarterial balloon catheter injury in athymic nude rats.[20] A similar role has been attributed to bFGF. It has been shown that the proliferation of SMCs is significantly reduced in animals that receive blocking anti-bFGF antibodies before balloon angioplasty.[22]

CONCLUSION

Many studies have supported a role for PDGF and bFGF in the genesis of MH and atherosclerosis. In our study we found that AVGs produce a greater quantity of growth factors. This phenomenon could represent the cause for the high incidence of stenotic changes seen in saphenous vein bypass grafts.

The results of our study support the view that pharmacologic intervention directed to reduce growth factor production might be beneficial in preventing stenotic changes in saphenous vein bypass grafts.[34]

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